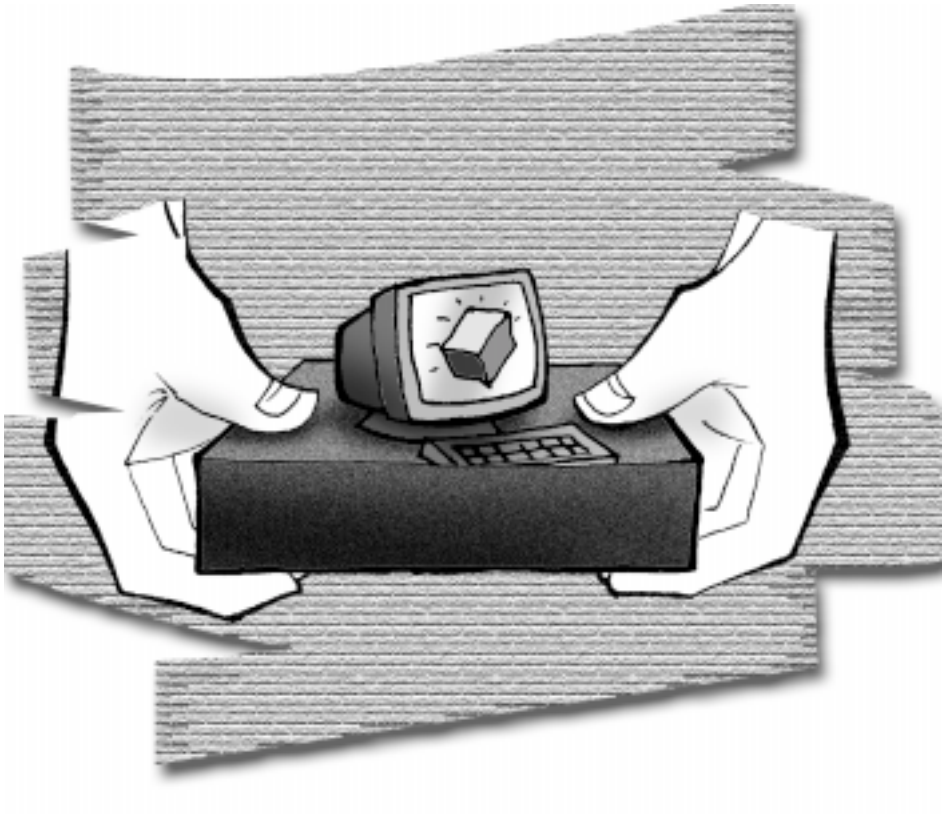
7 The use of molecular dynamics in *de novo* design: application to a transmembrane four-helix coiled coil



This chapter results from a collaboration with Dr. C. T. Choma (GBB) who designed and synthesised MEMBUN. C. T. Choma, D. P. Tieleman, D. Cregut, L. Serrano and H. J. C. Berendsen. Manuscript in preparation.

Summary
In this chapter we try to use molecular dynamics (MD) as a tool in the design process of a four-helix membrane protein (called MEMBUN). We ask two questions: 1. Can MD simulations say something useful about a design for a protein that has been created by some other means? 2. Can simulations help predict a detailed structure, given an amino acid sequence and a number of constraints on the structure? This question is in its most general form equivalent to the protein-folding problem, but a number of powerful constraints simplify the problem considerably.
To answer the first question, we have performed simulations of MEMBUN in a DMPC bilayer. MEMBUN is a dimeric oligopeptide consisting of two 62 residue peptides covalently linked by a disulfide bridge. It has been synthesised and has the correct mass, but no structural data is currently available. Analyses of a number of properties that can be expected to show instability show no reasons why the model would be unstable. Control simulations of the structurally related ROP protein confirm this conclusion. The main difference between MEMBUN and ROP is a reduced freedom of the sidechains in MEMBUN.
To answer the second question, we have used a combination of simulated annealing (SA) and MD on both ROP and MEMBUN. When the crystal (ROP) or model (MEMBUN) Cα atoms were used as constraints in generating a starting structure for MD, the simulations gave similar results as those starting from the full crystal structure or model. When the only constraint on the starting structure was an approximate orientation of four straight antiparallel helices, coiling was observed but the RMSDs from the MEMBUN model and ROP crystal structure were large.
Simulation and subsequent analysis of a model is fairly simple and can be done on a desktop workstation in a matter of weeks of computer time. Compared to the effort of design and synthesis this extra effort may well be justified. A more elaborate SA procedure combined with simulations, although potentially much more rigorous, is comparatively costly and complicated and emphasises the need for knowledge of accurate experimental constraints on the possible structures.

7.1 Introduction

Design and synthesis of proteins is a powerful method to study the complex interplay between the different interactions that determine protein tertiary structure and folding pathways [241–244]. Thus far, mostly water-soluble proteins have been designed and synthesised. They have several advantages over membrane proteins: the database of high-resolution structures to use in the design process is much larger for water-soluble proteins and synthesis or expression, purification, characterisation and structure determination are much easier. Nonetheless, it is of considerable interest to be able to study membrane proteins at the controlled level that design and synthesis can offer. It has been postulated that protein folding in membranes is fundamentally different from protein folding in solution, because the bilayer environment stabilises α-helices [232]. In the two stage model of Popot and Engelman [245], helices will form in the bilayer and subsequently aggregate to form the final tertiary structure. Design and synthesis would allow systematic studies of the factors that determine e.g. helix-bundle formation.

The proof of the pudding is in the eating, and in water-soluble proteins structure determination is the obvious way to validate a design. However, structure determination of membrane proteins is still a major undertaking, even for relatively small membrane proteins like glycophorin [246]. Because the synthesis of sufficient amounts of material for even biochemical characterisation is not trivial, it may be worth using theoretical methods like molecular dynamics to study a design, prior and complimentary to the synthesis and characterisation.

In this paper, we investigate how MD can be useful in the design process of membrane proteins. We ask two questions:

1. How can molecular dynamics test and possibly refine a design for a protein, regardless of how that design was made?

2. Given a design for a protein, can a combination of molecular dynamics and simulated annealing be used as a method to verify the design by trying to build a model based on rough ideas of the secondary and tertiary structure alone and comparing that to the original design?

To answer the first question, we have performed extensive simulations of the membrane protein MEMBUN, in the DMPC bilayer environment it was designed for. MEMBUN is designed as a covalently bound dimeric antiparallel four-helix bundle. It has been synthesised, and mass spectroscopy shows that it has the correct mass for the dimer [247, 248]. However, further characterisation has not yet been completed. We have analysed properties that might be used to distinguish between good and bad designs. As a reference, we

have also studied ROP [249]. This is a structurally related dimeric antiparallel water-soluble four-helix bundle. To estimate what happens when a protein is placed in an unfavourable environment, ROP was simulated both in water and in a lipid bilayer.

The second question is much more involved, and we do not pursue this question to the fullest possible extent. In its most general form, the question is equivalent to the protein folding problem. However, the lipid bilayer environment imposes a number of powerful constraints on the folding process [232]. In addition, we make a number of extra assumptions about the secondary structure and the orientation of the helices. This reduces the problem to the packing of four helices, which is related to a large number of studies in which the structure of membrane proteins was modelled by a combination of an atomic force field and experimental restraints on the possible structures (e.g. [148,201,203,250–254]). The main assumptions in the second approach are embodied in the initial positions of all Cα atoms. The other atoms are generated automatically by the procedure. For both ROP and MEMBUN we generated two sets of structures. In the first, the Cα atoms were taken from the crystal or model structure. This is an extreme case where basically the backbone is known already. In the second, the Cα atoms formed four antiparallel ideal helices. This assumes only a fairly rough knowledge of the structure of the protein. In both cases, the model building and simulated annealing steps are followed by long molecular dynamics simulations.

Although the simulated annealing procedure used here has been tested on a helix dimer [250,251], we are not aware of tests on larger proteins for which the modelled structure can be compared to an experimentally determined high-resolution structure. This makes it interesting to test the approach on ROP, in addition to using it as a method to predict the structure of MEMBUN.

7.2 Models and methods

7.2.1 The protein models

For ROP we used the 1.7Å crystal structure [249]. The situation for MEMBUN is more complicated, because there is no experimental structure. The exact design process and synthesis will be described elsewhere (C. Choma et al., in preparation), but we will outline the main features of MEMBUN here.

MEMBUN was designed and synthesized as a 63 residue mostly helical peptide. Two polypeptide chains are covalently bound by a disulfide bond, yielding a 126 residue protein. This disulfide bond severely limits the number of possible tertiary structures. The protein has a twofold symmetry and packs in an antiparallel fashion. The protein is modelled after ROP, but has been

adapted to a transmembrane orientation. The amino acid sequences for a monomer are given in Table 7.1.

Table 7.1. Amino acid sequence of the ROP and MEMBUN monomers.

ROP
Met-Thr-Lys-Gln-Glu-Lys-Thr-Ala-Leu-Asn-Met-Ala-Arg-Phe-Ile Arg-Ser-Gln-Thr-Leu-Thr-Leu-Leu-Glu-Lys-Leu-Asn-Glu-Leu-Asp Ala-Asp-Glu-Gln-Ala-Asp-Ile-Cys-Glu-Ser-Ile-His-Asp-His-Ala Asp-Glu-Leu-Tyr-Arg-Ser-Cys-Leu-Ala-Arg-Phe
MEMBUN
Ace-Cys-Gly-Ser-Pro-Asp-Gln-Val-Trp-Leu-Asn-Val-Leu-Val-Leu Val-Ser-Leu-Leu-Asn-Val-Leu-Val-Ser-Leu-Tyr-Thr-Ala-Gln-Lys Ala-Lys-Asn-Gly-Gly-Asp-Ala-Glu-Leu-Glu-Asn-Ala-Val-Tyr-Leu Asn-Ala-Leu-Val-Ser-Leu-Leu-Asn-Ala-Leu-Val-Ser-Leu-Trp-Thr Ala-Lys-Asn-Pro-Gly-NH2

MEMBUN was designed to be thermodynamically stable in three regions: it has a hydrophobic exterior in the middle of the bilayer, designed as a true integral membrane protein, hydrophilic ends, designed as a water-soluble protein, and tryptophans and tyrosines flanking the bilayer, designed to be stable at the bilayer-water interface.

In water-soluble coiled coils, the interior of the bundle consists of hydrophobic residues that pack in a regular pattern [244, 255, 256]. However, in MEMBUN a H-bond network between Ser and Asn residues was designed into the two central heptads of the bundle, where the bundle is most deeply embedded in the hydrocarbon core of the bilayer. This H-bond "zipper" restricts the axial rotation of the four helices. The major contribution to bundle stability comes from hydrogen bonding in the interior, as opposed to hydrophobic packing interactions and the hydrophobic effect in water-soluble coiled coils. In Fig. 7.1, a graphical view of ROP and MEMBUN is given.

7.2.2 Simulated annealing and restrained molecular dynamics

The simulated annealing protocols (using X-plor with the Charmm param19 force field implemented in X-plor [257]) we used are based on the method described by Nilges and Brünger [250, 251]. Similar protocols have also been used in studies on membrane channel models by Sansom and coworkers [193, 203, 258]. There are basically four steps in this protocol:

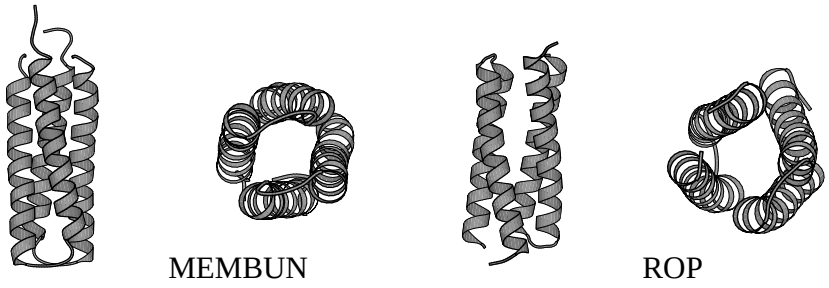


Figure 7.1. Molscript [79] snapshots of the MEMBUN model and ROP crystal structure.

1. Generate a Cα template of the structure. Initially, all atoms in a residue are superimposed on the Cα atom. This Cα template embodies most of the assumption made about the structure. We used two different templates for both ROP and MEMBUN. The first is just the Cα coordinates of the crystal structure for ROP and the model for MEMBUN (the SA1 systems in table 7.2). This can be considered an upper limit on the accuracy of this method. For the second template, we generated an antiparallel four-helix bundle of straight helices, assuming an ideal geometry of 1.5 Å helical rise per residue and an inter-helix distance of 8.4 Å. The four helices were rotated such that the hydrophobic side for ROP and the hydrophilic side for MEMBUN face roughly inward (the SA2 systems in table 7.2). The vertical orientation in both cases is determined by the inter-helical loops. In the case of MEMBUN, the disulfide bond is present during the entire modelling phase.

2. The Cα atoms are kept fixed during a simulated annealing run starting at 1000 K. Initially, the force constants for all bonded interactions are scaled down to a fraction of their normal strength. In the course of a 2 ps run at 1000 K, first the bonds and angles, and with a small lag the dihedrals and improper dihedrals, are increased in strength until they reach their normal values. When the bonded terms have reached their normal values, we introduce a small repulsive Van der Waals term and cool the system to 500 K. The resulting structure is energy minimized. This process is repeated 5 times, resulting in 5 structures.

3. These 5 structures are used as starting point for the next step. Initially, harmonic restraints are imposed on all Cα atoms of the helices. These restraints are gradually relaxed as the system is cooled from 500K to 300K. There still is a weak restraining potential that prevents the helices

Table 7.2. Overview of the simulations.

system name	description	length	atoms
1. MEMBUN	MEMBUN in DMPC bilayer	2 ns	10786
2. ROPlip	ROP in DMPC bilayer	2 ns	10745
3. ROP	ROP in water	2 ns	18497
4. ROPSA1	SA model 1 of ROP in solvent	1 ns	16511
5. ROPSA2	SA model 2 of ROP in solvent	1.8 ns	19487
6. MEMBUNSA1	SA model 1 of MEMBUN in DMPC	2 ns	10786
7. MEMBUNSA2	SA model 2 of MEMBUN in DMPC	7 ns	8467

from moving too far apart. In addition, backbone hydrogen bonds are strengthened by restraints. Electrostatics are also slowly introduced during this stage. By repeating this stage 5 times we create an ensemble of 25 structures

4. The 25 structures are simulated in vacuum for 5 ps with the full potential form, including restraints on the helices, the backbone helical hydrogen bonds and a distance dependent dielectric constant. The 25 resulting structures are averaged by best fitting them on top of each other and calculating the average position of the coordinates. The resulting structure is energy minimized and used as starting point for a long MD run.

7.2.3 Full simulations

The starting structure for the DMPC bilayer was made from an equilibrated bilayer of 64 DPPC lipids [178]. The last two carbons of both tails of all lipids were removed and the two bilayer leaflets were translated 0.4 nm toward each other. The resulting DMPC bilayer was simulated for 50 picoseconds, after which a cylindrical space was created by removing 11 lipids per leaflet and applying a repulsive cylindrical potential. MEMBUN was placed in the hole with the tryptophan residues flanking the hydrophobic core of the protein at the acylchain-glycerol interface. Water was re-added and removed from the bilayer interior. Two sodium ions were added, by replacing water molecules at the positions with the lowest Coulomb potential, to make the system electrically neutral. The resulting system was energy minimized and used as starting point for a simulation. The same starting structure was used for a simulation of ROP in a DMPC bilayer, by replacing MEMBUN with ROP. The models obtained from simulated annealing were used in additional simulations, by replacing MEMBUN with the models. In all bilayer simulations the same simulation para-

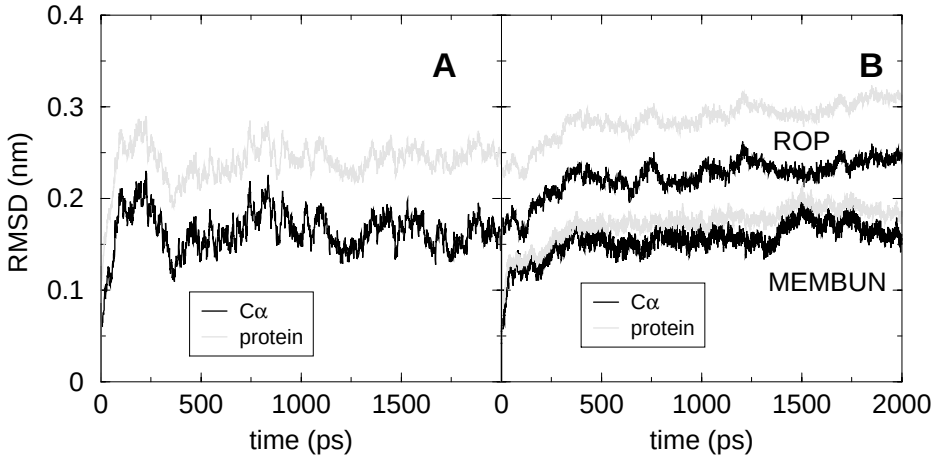


Figure 7.2. RMSD as function of time, fitted on the crystal or model structure Cα atoms for **A** ROP in water; **B** for ROP and MEMBUN in DMPC.

meters and force field were used as in chapter 4, except the long range cutoff was only 1.4 nm due to the smaller boxsize. ROP was solvated in SPC water with 8 Na^+ ions and simulated using standard procedures [259]. Bonds were constrained using LINCS [260].

7.3 Results

7.3.1 Challenging MEMBUN: full simulations

The most obvious candidate properties from a simulation that might indicate the stability of a protein are a number of structural properties that describe the change in structure over time, including the RMSD, secondary structure, number of hydrogen bonds, the radius of gyration and the solvent accessible surface.

The RMSD curve for ROP in water (Fig. 7.2A) is fairly typical, with a fast initial rise and after a few hundred ps a stable RMSD of ~0.15 nm for the Cα atoms, 0.25 nm for all atoms. MEMBUN has an even lower RMSD for the Cα atoms in the bilayer (Fig. 7.2B). Surprisingly, the difference between the RMSD for all atoms and just the Cα atoms is very small for MEMBUN, much smaller than for ROP. It seems the conformational freedom of the sidechains is more limited in MEMBUN than in other proteins. If ROP, with its typical hydrophilic

exterior, is placed inside a lipid bilayer, the RMSD does not stabilise and keeps increasing to much higher values than for ROP in water. Although this is only one number, it does indicate that the simulations have some power to distinguish between a "normal" and an unfavourable situation.

Both ROP and MEMBUN have a stable secondary structure over 2 ns (Fig. 7.3). However, the first 10 residues of MEMBUN do not form a helix, although the last of them were designed as helix. The rest forms the loop that connects to the other monomer via the disulfide bond (Cys2). All 10 residues reside in the headgroup region of the bilayer where there are many water molecules and lipids that can form hydrogen bonds. Residues 31 to 35 form a connecting loop with the next helix. Residues 61 to 66 are the C-terminus of the first monomer; Cys68 and the next few residues are part of the disulfide bond area. Residues 96 to 99 are the short connecting loop between the two helices of monomer two. Finally, the last few residues of the C-terminus of monomer two are in the headgroup region and do not form a helix either. In water, ROP consists of four completely stable helices joined by short loops.

Fig. 7.4 shows an overview of the hydrogen bonding for MEMBUN. The total number of $n \rightarrow n+4$ hydrogen bonds increases somewhat initially, to become stable after a few hundred ps. Fig. 7.4B shows the total number of hydrogen bonds within MEMBUN. This number remains basically constant during the entire simulation, including the starting model. A number of new hydrogen bonds form between MEMBUN and lipids, while the number of hydrogen bonds with water decreases somewhat. This is simply a result of placing MEMBUN in the bilayer environment.

Other structural properties such as the hydrophilic and hydrophobic solvent accessible area do change less than a few % for both systems (data not shown). A rough picture of the degree of sidechain packing of the protein is given by the radius of gyration. The total radius of gyration of ROP in water remains constant during the simulation. For MEMBUN in a bilayer the radius of gyration decreases by less than 2%. If ROP is placed in a bilayer, its radius of gyration increases slightly, from 1.44 nm to 1.48 nm.

A characteristic property of proteins is that they possess a small number of collective degrees of freedom that account for the majority of the motion [186, 261]. A principle component analysis on the MEMBUN and ROP trajectories yields the eigenvalues and eigenvectors that describe the motion of the protein [186]. The largest eigenvalues belong to eigenvectors that describe collective motions with a large amplitude. The cumulative total of the first n eigenvalues shows how much of the total motion is accounted for by the n corresponding eigenvectors. This total is plotted in Fig. 7.5 for MEMBUN and ROP. The precise shape of the curve is irrelevant, and large fluctuations occur between different proteins and even different runs of the same pro-

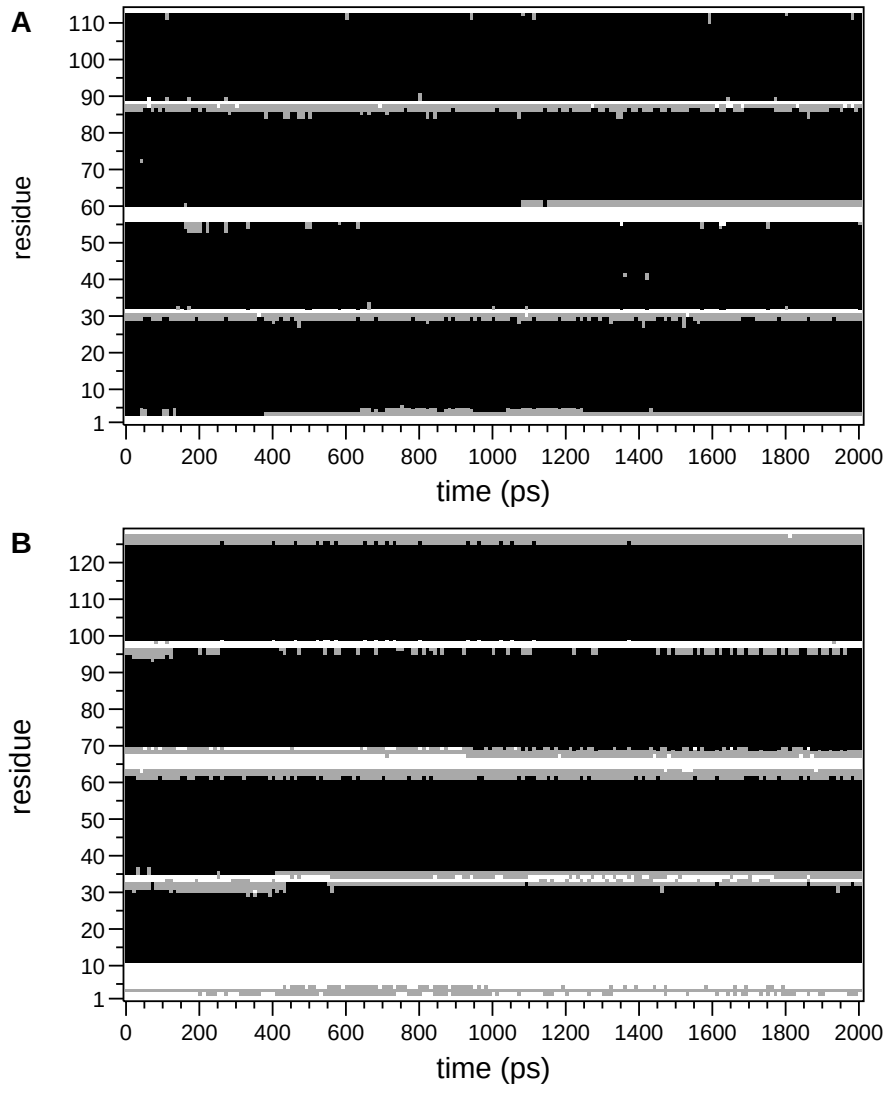


Figure 7.3. Secondary structure as function of time for **A** ROP in water; **B** MEMBUN in DMPC. White is coil, light grey turn, dark grey 3-10 helix and black α-helix. Secondary structure was calculated with DSSP [146].

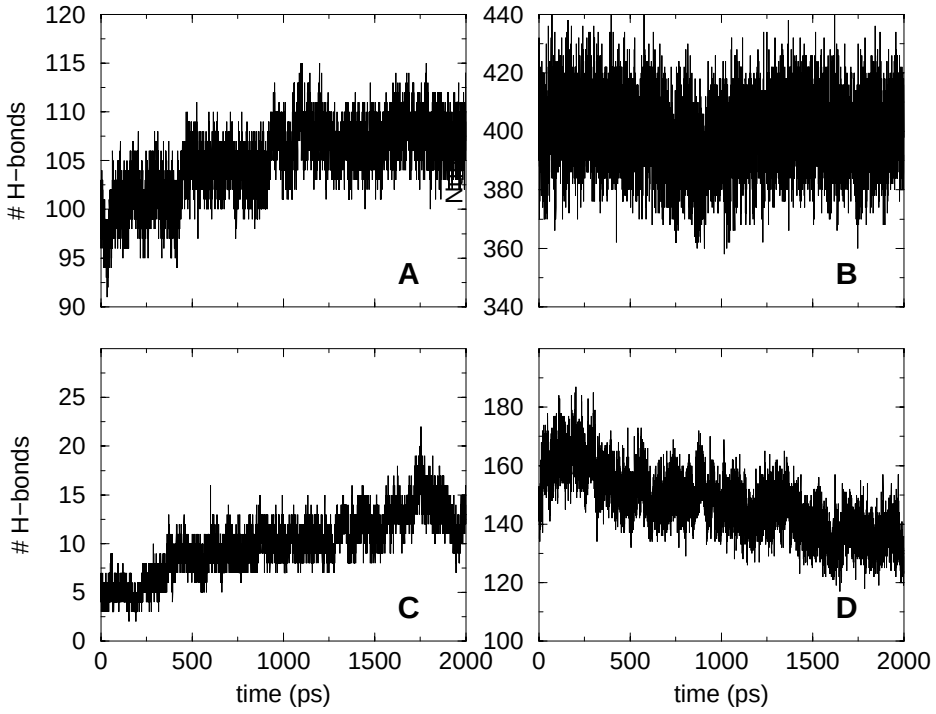


Figure 7.4. Overview of hydrogen bonding in MEMBUN. **A** Hydrogen bonds between residues n and $n + 4$. **B** All hydrogen bonds within the protein. **C** Hydrogen bonds between the protein and the lipids. **D** Hydrogen bonds between the protein and water

tein. However, it is clear that MEMBUN has a small number of a few dozen eigenvectors that describe most of its motion. A soft polymer would have a more even distribution of eigenvalues, and from this point of view MEMBUN behaves like a protein.

The secondary and tertiary structure of a protein imposes significant constraints on the possible motions. It can be expected that a designed protein does not have the same degree of intrinsic constraints as a native protein, because these constraints are formed by many contributing factors that are difficult to pinpoint. A lack of powerful constraints becomes visible in an essential dynamics analysis as a collective mode that is not reproducible across simulations or parts of simulations [262, 263], provided the simulations are long enough to define the essential subspace. If the inner product matrices of the eigenvectors determined from two different simulations (or, like in

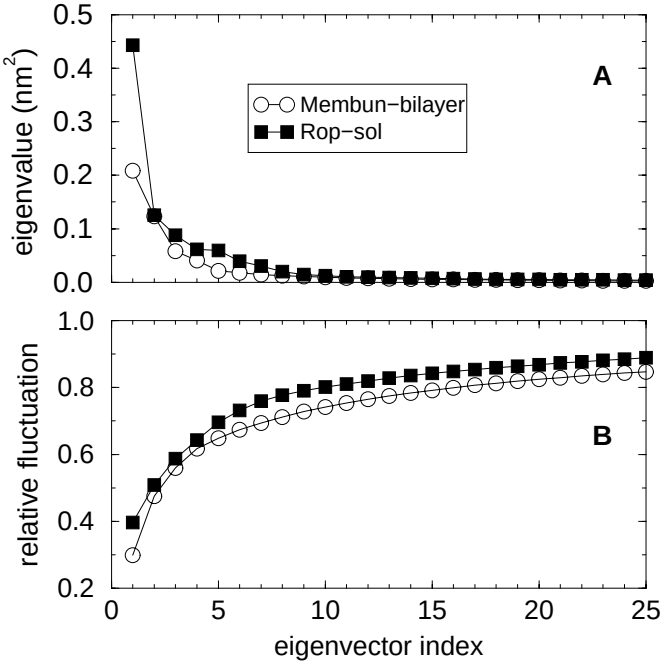


Figure 7.5. A Eigenvalues of the first 25 eigenvectors. **B** Relative subspace positional fluctuation with respect to the total positional fluctuation as function of the number of contributing eigenvectors.

this case, two parts of one simulation) are plotted, there will be strong peaks at locations far away from the diagonal due to collective modes of motion present in one but not in the other simulation. However, this is not the case for ROP or MEMBUN (Fig. 7.6). If anything, the constraints on MEMBUN's structure are more pronounced than on ROP. This indicates that from a dynamical point of view, the MEMBUN model does behave like a real protein.

7.3.2 Simulated annealing and subsequent simulations

The primary result from the SA runs is four sets of 25 structures, 2 for MEMBUN and 2 for ROP. A random selection of those is plotted in Fig. 7.7 for ROP and in Fig. 7.8 for MEMBUN. If the Cα atoms are used as template, the overall coiled coil structure is mostly conserved and the main differences for ROP are at the ends of the helices and in the long sidechains extending away from the

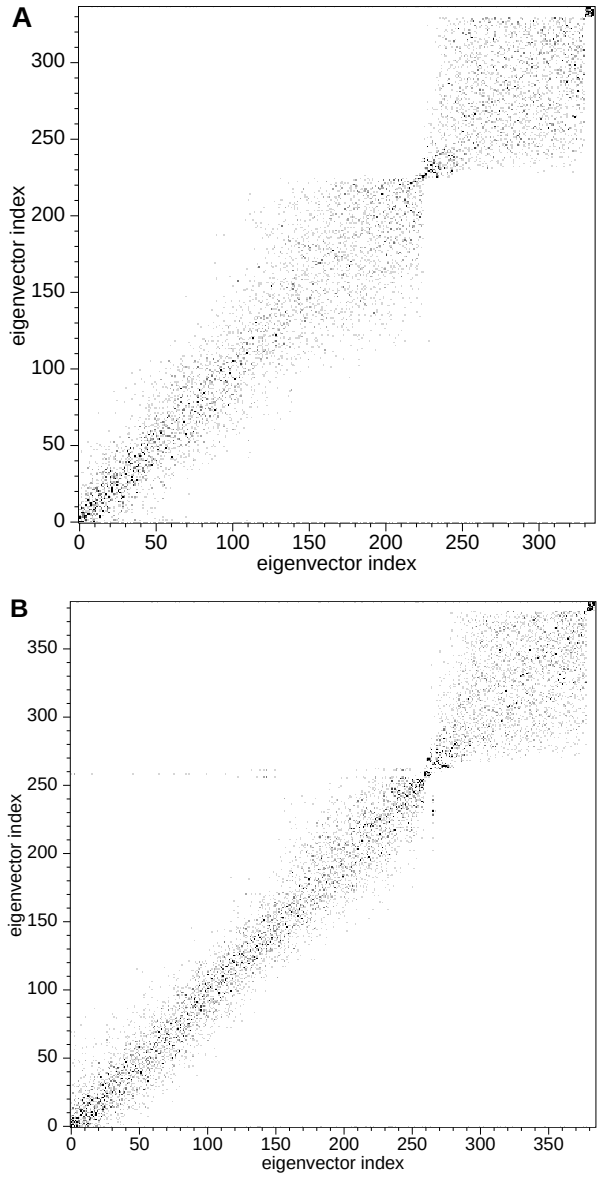


Figure 7.6. Squared inner product matrices for **A** ROP and **B** MEMBUN, calculated from the first and the last nanosecond of each trajectory.

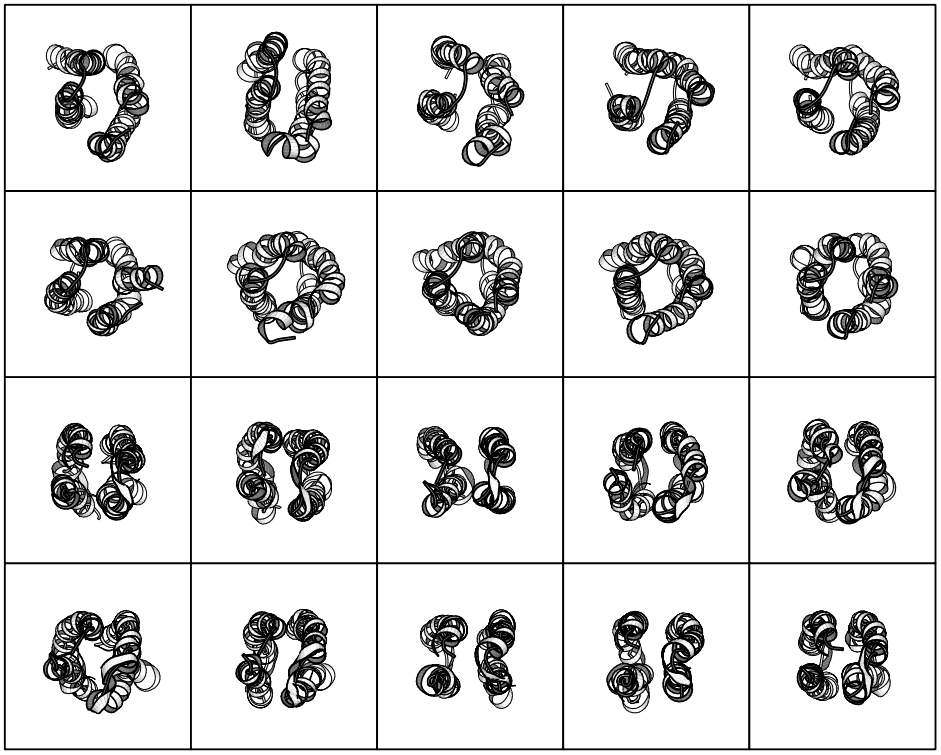


Figure 7.7. Structures generated by simulated annealing starting from the Cα atoms of the ROP crystal structure (upper 10) and from four straight helices (lower 10). For each case 10 out of a total of 25 structures are shown.

helix. The average RMSD from the mean structure of the ensemble is 0.19 nm for backbone atoms and 0.25 nm for all heavy atoms in this case. The RMSD for the structures that assumed straight helices is comparable, 0.19 nm for the backbone and 0.25 nm for all heavy atoms. Several of these structures start coiling, but not all of them.

The RMSD from the mean structure for MEMBUNSA1 is considerably lower, 0.13 nm for the backbone atoms and 0.17 nm for all heavy atoms. Interestingly, these values are not only much lower than for ROP, but they are also much lower than for the SA2 model (0.18 nm for the backbone, 0.23 nm for the heavy atoms). It appears that packing in MEMBUN is more restricted than in ROP. A significant change in the backbone structure causes a looser packing, resulting in a larger RMSD within the ensemble.

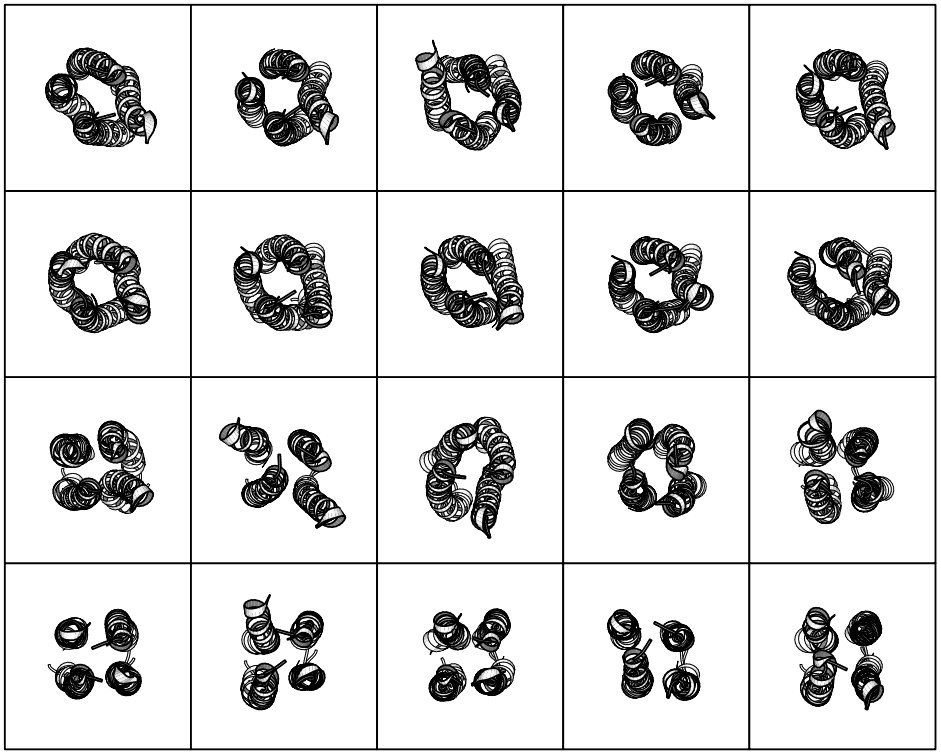


Figure 7.8. Structures generated by simulated annealing starting from the Cα atoms of the MEMBUN model (upper 10) and from four straight helices (lower 10). For each case 10 out of a total of 25 structures are shown.

The rationale for the extended MD simulations in solvent or DMPC is that the environment of the protein is taken into account correctly. It is interesting to see if MD simulations can bring the structure that is generated from the SA procedure closer to the "real" structure. In the case of ROP, this is the crystal structure. In Fig. 7.9A the RMSD vs. time for ROP is plotted for ROPSA1 and ROPSA2. In both cases the RMSD shows no tendency to approach the crystal structure. In the first case, the RMSD is somewhat higher than for the run starting from the crystal structure. The final structure after 1 ns of simulation in water is still a coiled coil (Fig 7.10), and the main differences with the crystal structure are found at the ends of the protein. In the second case, the RMSD with respect to the crystal structure is very high, close to 0.4 nm. The final structure of this run shows that the coiled coil is mostly lost and the helices

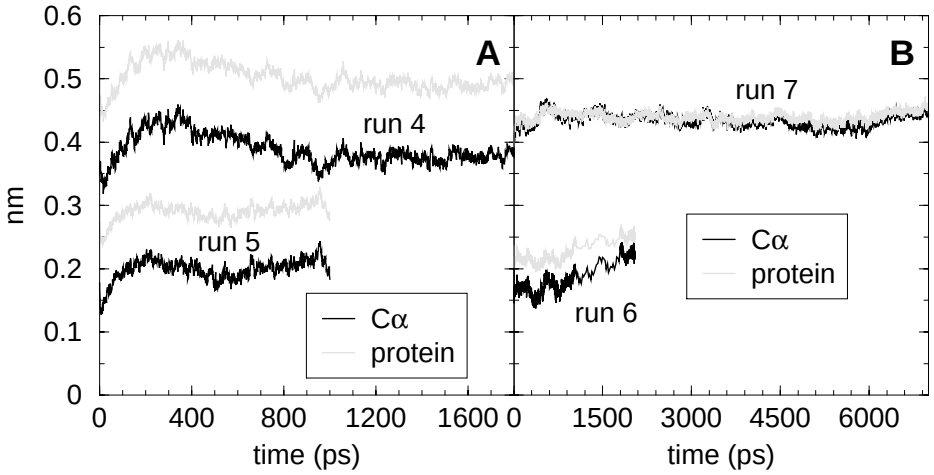


Figure 7.9. RMSD as function of time with respect to **A** the ROP crystal structure and **B** the MEMBUN model.

are no longer packed as tightly (Fig. 7.10). This emphasises the importance of accurate experimental constraints on model building procedures.

With the results for ROP in mind, it is doubtful that an extended simulation of an inaccurate starting model (MEMBUNSA2) will result in an improved prediction. Starting from the model Cα coordinates, the MEMBUNSA1 run stays relatively close to the model structure (Fig. 7.9B). The second run, which is quite long by current standards, has a RMSD of 0.45 nm for both backbone and all atoms compared to the MEMBUN model. It shows no trend to move closer toward the model structure. However, this has little predictive power because we do not know the true structure of MEMBUN. It does demonstrate the limitations in sampling inherent in MD [264]. The final structures after 1 ns for MEMBUNSA1 and 7 ns for MEMBUNSA2 are given in Fig. 7.10.

7.4 Discussion

7.4.1 Stability of MEMBUN

Structural and dynamic properties show no indication that MEMBUN is unstable. The essential dynamics analyses demonstrate that it has internal constraints on the structure so that most of its motion can be described by just a few collective degrees of freedom. Structural properties such as the RMSD

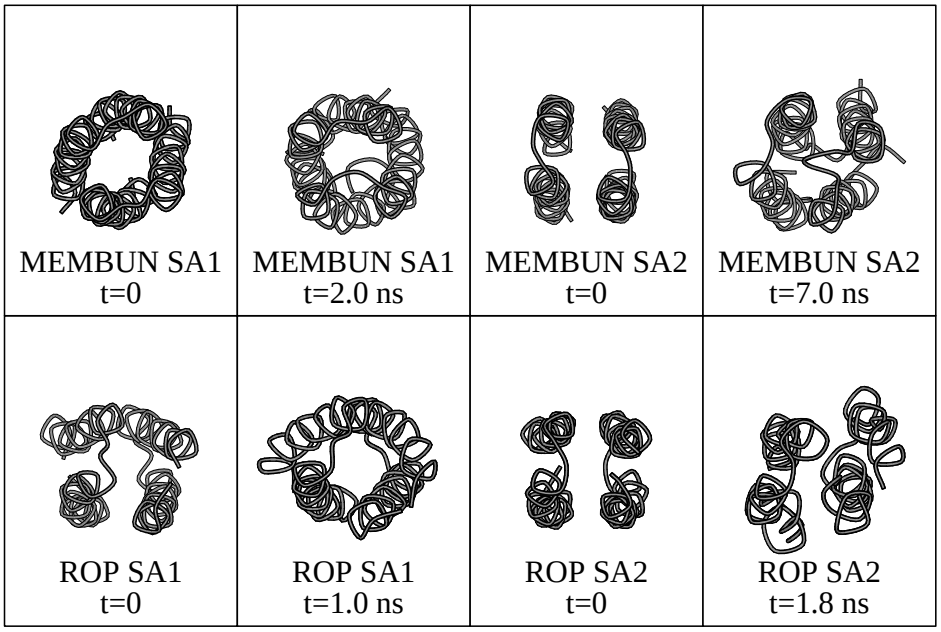


Figure 7.10. Cα traces of the starting structure and final structure of runs 4–7.

and secondary structure indicate a stable structure, as opposed to ROP in DMPC, which does not seem to be stable. A difference with ROP is the limited freedom of the side chains, as indicated by the small difference between the backbone and all-atom RMSD. This might be either favourable (increased packing so increased Van der Waals and hydrogen bonding energy) or unfavourable (reduced entropy for the sidechains), but to decide between the two we need a more detailed knowledge of the factors that influence helix aggregation in bilayers.

The SA modelling yields a number of structures but is only accurate enough to be interesting when there are detailed experimental constraints on the structure. In the case of MEMBUN, no such data is available. The generated structures indicate that the straight MEMBUN helices are likely to coil, even if the backbone structure is different from the intended one. With the model Cα atoms as initial template, the difference between the generated structures is much smaller for MEMBUN compared to ROP, which correlates with the low RMSD for the sidechains in the simulation of the model. The main differences with the model are at the ends of the helices, which is encouraging.

7.4.2 Assumptions and limitations

We have made a number of assumptions. The most serious one is that MEMBUN will actually insert as a tetrameric helix-bundle into a bilayer. However, it seems that as long as there is a continuous lengthwise hydrophobic face covering over 50% of the helix, helix formation and transversal in membranes is rather indifferent to sequence [265, 266]. Although helix formation and aggregation inside the bilayer seem likely, it is possible that the protein will form random aggregates outside the bilayer and possibly precipitate. A second possibility is that MEMBUN will associate with the bilayer surface, and form a cross-shaped structure with the hydrophobic side facing the bilayer, the hydrophilic side facing the solvent. Finally, it is possible that once inserted into a bilayer the protein will not form a four-helix bundle. However, this seems unlikely due to the disulfide bond that keeps the four helical segments together.

This assumption of insertion and aggregation cannot be tested by simulations of the type we described, and we have to wait for biochemical data to determine if these assumptions are valid. Our goal here is to determine whether MD can contribute to the design process. A number of additional assumptions are implicit in the modelling and simulation procedures. MD does can not guarantee stability, mainly because of the limited time scale of simulations. The best we can do is to monitor structural properties, and in the case of MEMBUN these are stable. In contrast, a wrong model for an alamethicin channel (chapter 5) is not stable, and ROP in DMPC is not stable either. The SA procedure depends critically on experimental data to incorporate. If the Cα atoms are used as starting point, the structure for ROP is not unreasonable, although it is less accurate than the structure that was obtained for a two-helix leucine zipper [251]. For membrane proteins, more elaborate searching algorithms than the two simple Cα templates we have used here are commonly used [203, 252–254, 258]. This increases the accuracy of the modelling greatly, but is not feasible for a design for a membrane protein.

7.4.3 Outlook

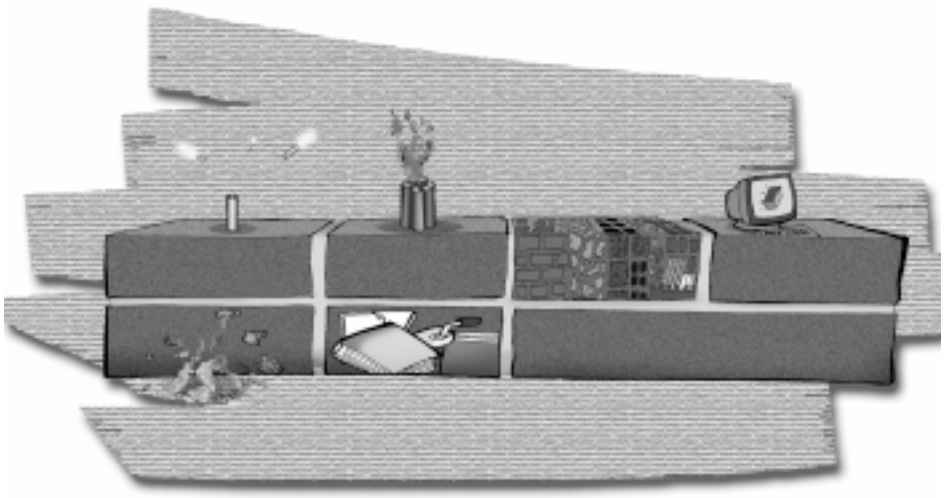
In spite of these assumptions and limitations, simulations and modelling with an atomic force field are likely to be the only method that can give detailed information on the feasibility of a model before it is actually synthesised or expressed. If in a simulation helices unfold, the structure drifts far away from the model, or the radius of gyration changes significantly there is a major problem with the design and it can be changed before attempting to synthesise it. If there are small problems the models may give possible structures, and the spread in the models may indicate the tightness of the design.

It is becoming more and more common that model building or protein structure prediction methods yield a series of possible structures of (membrane) proteins [241, 258, 267, 268]. Although MD cannot be used to distinguish between a large set of quite different structures, protein folding potentials can only reduce the set of possible structures to a smaller subset. It is of great interest to develop methods that can help distinguish between the few different models, from protein folding potentials or modelling based on experimental constraints, that cannot be excluded based on other data. In this context, MD simulations may be useful if we know which properties can be used to distinguish between good and bad models.

Simulations of small membrane proteins like MEMBUN can be performed on desktop workstations in a few weeks using modern MD software. In the case of MEMBUN this is a relatively small effort compared to the design, synthesis and characterization. If such simulations can dismiss a model before a synthesis is attempted they are easily worth the effort. In the case of MEMBUN, the additional simulations of ROP were useful to have a reference, but as more simulations of membrane proteins become available those can serve as references as well. The SA modelling is comparatively complicated, although potentially much more powerful. However, it depends critically on constraints in the structure.

The next step for MEMBUN is further structural characterization, which is currently commencing. From a computational point of view, it would be interesting to have a test system consisting of small membrane proteins for which there are known faulty and known good models, verified by experiment. Such a system would make it possible to further test and refine the simulations, and study the factors that determine packing and aggregation both theoretically and experimentally [269].

Conclusion: the use of molecular dynamics to study models of biological membranes



Biological membranes are fascinating structures. They consist of a complex mixture of lipids and proteins, which together perform a wide variety of tasks: from being a wall between inside and outside of cells and organelles to regulating transport of proteins, ions, energy and information. Many important processes can be studied, both experimentally and theoretically, in greatly simplified membrane models that only have a few components. Molecular dynamics simulations provide a window on the microscopic properties of such simplified models. In spite of the limitations in time and length scale that are inherent to MD, many properties of lipids, membrane proteins and water can be studied with simulations.

Pure lipids in lipid bilayers have been extensively studied, and they remain an important test system for further improvements in algorithms and force

fields. Simulations of lipid bilayers including membrane proteins are a more recent development. I hope the simulations in this thesis have contributed to developing this particular area. There are many ways to extend the current simulations.

OmpF was simulated in an open conformation, which is its natural state at neutral pH and low electric field. However, single channel recordings show that the protein exhibits gating behaviour, and AFM measurements at low pH or high electric field show a reproducible, closed conformation of the porin. There have been long discussions on the nature of these conformational changes, but it is not clear what their molecular basis is. Simulations at low pH might elucidate the closed conformation seen by AFM, while simulations of mutants or with an applied electric field may shed more light on the observed gating behaviour.

Alamethicin is an example of a peptide that has anti-microbial activity by perturbing a cell membrane, eventually causing cell lysis. As such, it can be regarded as a model for a large class of anti-microbial peptides and toxins. Many of these peptides have a potential use as food preservative or antibiotic. We have simulated only a few of the steps that lead to the death of a bacterium. Several interesting steps are missing: binding of alamethicin to a membrane, insertion of a single helix into the bilayer, aggregation of single helices into channels, transport of ions through these channels, and changes in conductance state by changes in the number of helices that form a channel. These steps all pose significant methodological problems that we are currently trying to solve, with the detailed simulations in this thesis as reference.

Alamethicin and porin were simulated under equilibrium conditions. In real cells, and in many experiments, there is an important role for ion, pH, and osmotic gradients across the membrane. Incorporating these factors into the models will be a great challenge for future work. At the moment it is an unsolved problem how to make the link between the atomistic models in simulations and the observed current-voltage curves from electro-physiological measurements. Both the alamethicin channel model and the porin system would make useful starting points to approach this problem. In addition, there is now at least one high-resolution structure of a potassium channel available, and it seems likely more will follow in the future. Ultimately, a combination of theoretical and experimental techniques may find a detailed explanation of the basic processes of voltage gating, selectivity and fast transport in ion channels.

In the last chapter, we have tried to use molecular dynamics simulations as a tool in the design of membrane proteins. It is relatively simple to

take a model and test whether it is stable (on a nanosecond time scale) in the environment it was designed for. Although this does not guarantee a synthesis will be successful, it may detect a wrong model for the moderate cost of a calculation of a few weeks on a modern pc or workstation. It is early day for the design and synthesis of membrane proteins, and it is worthwhile to look at simulations of designed proteins again when more of them are available. It would be particularly useful to have a series of designs, some of which are known to be valid, while others are known to be faulty. Of course, the procedures used in chapter 7 are similar to those used to create models of membrane proteins based on incomplete structural data, including models of the alamethicin channel and influenza M2. The results of these procedures emphasise the importance of including as much experimental information as possible in the models.

A science thesis is not a best-seller, and I have seen pessimistic estimates for the number of readers of a thesis that go as low as four. Because I am basically an optimistic person, and hope that some people doing experimental work are still reading this, I would like to point out the following: although at the moment computer simulations are usually done by people working in theory groups, often on seemingly exotic systems, there are two reasons why this is likely to change in the future.

The first is that nowadays simulations of realistic systems can be run on desktop computers. A simplified lipid bilayer with 12 sites per lipid that needs to be run on the local supercomputer is not likely to appeal to someone doing experimental work on lipid bilayers. However, if a realistic model of a bilayer can be simulated and analysed on a desktop computer in a matter of weeks, it becomes more interesting to include a computational part in biochemical research. The second reason is that the learning curve for using modern molecular dynamics programs and protocols is not as steep anymore as it once was. Combined, modern computers and modern programs can make modelling and simulations a worthwhile tool in general biophysics and biochemistry.

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